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Isolation, culture, and immunocytochemical characterization of epididymal epithelial cells from pubertal and adult rats

(acidic epididymal glycoprotein/collagenase/principal cells/DNA synthesis/autoradiography)

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ABSTRACT **A method for the isolation and culture of epididymal epithelial cells obtained from pubertal and old adult rats is described. This method permits the establishment of primary cultures of these cells in monolayers from aggregates isolated from whole epididymides and major epididymal anatomical segments (caput, corpus, and cauda) after trypsin and collagenase digestions. A large number of cultured epididymal cells retain a differentiated function as demonstrated by the immunocytochemical and radioimmunoassay finding of acidic epididymal glycoprotein, a spermatozoa-coating protein secreted by the principal cells of rat epididymis. The proliferative potential of cultured epididymal cells obtained from pubertal and old adult donors can be documented by [^{3}H]thymidine labeling and mitotic indices without significant loss of gene expression for acidic epididymal glycoprotein. Results of this study demonstrate that epididymal epithelial cells, consisting of a predominant population of principal cells, can be isolated, cultured, and maintained for up to 3 months.**

Knowledge of the factors that regulate epididymal epithelial cell function is relevant to the understanding of post-testicular events related to male fertility. There is general agreement that the highly convoluted epididymal duct provides an appropriate environment for spermatozoa maturation and survival. Experimental evidence has been presented in support of the androgen dependency of the epididymis. In fact, androgen withdrawal results in regressional changes of the epididymal epithelium (1–3) and in a rapid loss of spermatozoa viability within a few days (4, 5).

Androgen-binding protein, a secretory product of testicular Sertoli cells, is transported to the epididymis via testicular fluid in the form of an androgen–protein complex (6). It has been proposed that androgen-binding protein provides an adequate concentration of androgens (7) required for the maintenance of epididymal structure and function (8, 9).

Evidence has also been presented suggesting that secretory proteins released by the epididymal epithelium into the lumen of the duct contribute, together with cyclic nucleotide metabolic factors, to the acquisition of spermatozoa motility (10) and to spermatozoa surface modifications (11) as the male gamete traverses diverse anatomical segments of the mammalian epididymis. For instance, bovine forward motility protein, a protein present in epididymal and seminal fluids, is involved in the initiation of spermatozoa motility (12). Furthermore, acidic epididymal glycoprotein, an androgen-dependent secretory product originating in the principal cells of the rat caput and corpus epididymis, binds to spermatozoa as they pass through the caput (13). Other rat epididymal glycoproteins have been identified as components of the spermatozoa plasmalemma (14).

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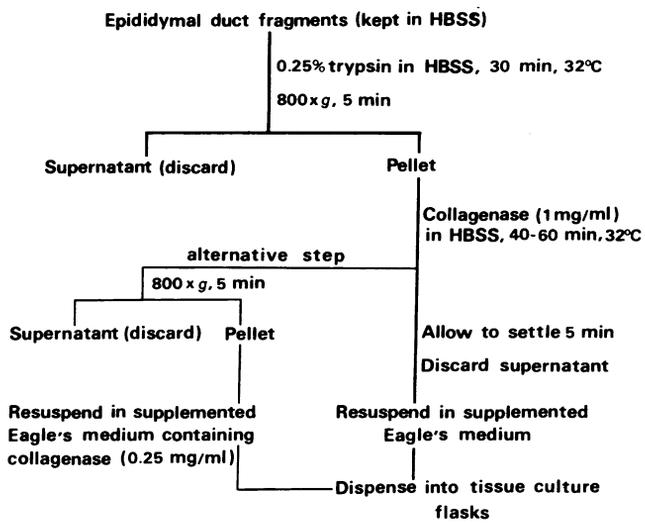

FIG. 1. Steps in the culture of epididymal epithelial cells from rat epididymis. HBSS, Hanks' balanced salt solution.

To elucidate the function of the epididymal epithelium in particular, efforts have been directed toward: (*i*) development of methods for separation of homogeneous populations of viable epididymal cells (including epithelial cells) by using unit gravity sedimentation techniques (15–18) and (*ii*) development of primary cultures of epididymal epithelial cells. In this paper, we report a procedure for the isolation and culture of rat epididymal epithelial cells. The predominant cell type present in cultures of these cells has been characterized by immunoperoxidase and radioimmunoassay techniques using a specific antiserum to acidic epididymal glycoprotein.

MATERIALS AND METHODS

Isolation and Culture of Rat Epididymal Epithelial Cells. The procedure is summarized in Fig. 1. Epididymal epithelial cells were isolated from epididymides of 20- to 22-day- and 20-month-old rats (Charles River Breeding Laboratories). Epididymides were dissected free of fat and connective tissue under sterile conditions in Hanks' balanced salt solution. Whole epididymides and epididymal segments [caput, zones 1–3 according to the Reid and Cleland classification (19); corpus, zone 4 and the proximal part of zone 5; and cauda, distal part of zone 5 and zone 6] were dissected out, minced into small fragments (about 2–3 mm^3) and transferred to 0.25% trypsin (pancreatic type II, Sigma, no. cat. T-8128) in Hank's balanced salt solution (pH 7.6) (5 mg of tissue per ml of solution). After incubation at 32°C for 30 min in a thermo-bath shaker (60 cycles/min), the

sample was centrifuged at low speed (800 × *g*, 5 min), the supernatant was discarded, and the pellet was suspended in collagenase (1 mg/ml equivalent to 146 units/mg of solid, Sigma, cat. no. C-0130) in salt solution (pH 7.6) (5 mg of tissue per ml of solution). After incubation at 32°C for 40–60 min in a thermobath shaker as above, the sample was allowed to settle for 5 min, the supernatant was discarded, and the sediment, consisting mainly of epididymal epithelial cell aggregates and traces of the collagenase solution, was suspended in Eagle's minimal essential medium supplemented with nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), glutamine (final concentration, 4 mM), 5α-dihydrotestosterone (1 nM), 10% fetal bovine serum, and antibiotics (penicillin at 100 units/ml and streptomycin at 100 μg/ml).

Residual collagenase in the cell sediment (about 0.25 mg/ml equivalent to about 35 units/mg of solid) becomes a transient component of the tissue culture medium during the first 12–24 hr of culture. At that concentration, collagenase has a growth inhibitory effect on nonepithelial cells (20) without affecting epididymal epithelial cells viability. An alternative centrifugation step at low speed (800 × *g*) for 5 min can be carried out after the collagenase digestion step (Fig. 1). The pellet is resuspended in supplemented Eagle's medium containing collagenase (0.25 mg/ml). Single and aggregated epididymal epithelial cells are plated in 25-cm^2 tissue culture flasks at a cell density of 1×10^4 cells per ml and incubated in a humidified CO_2/air incubator at 32°C.

An additional procedure which prevents the possible contamination of the primary cultures with fibroblasts and smooth muscle cells can be carried out. The plated sample is allowed to settle in the tissue culture flasks for about 8–12 hr. Then, the supernatant containing aggregates of epididymal epithelial cells is transferred to a new culture flask. Under these conditions, nonepithelial cells attach very rapidly, thus leaving epididymal epithelial cells aggregates in suspension. When transferred to new culture flasks, the aggregates attach to the substrate and establish a colony-like growth. Epididymal epithelial cells attach to the substrate within 24 hr and become flat. The attachment of epididymal epithelial cells aggregates isolated from 20-month-old rats is slower (24–36 hr), probably due to contaminating spermatozoa. Trypsin-resistant spermatozoa and residual heads and tails in epididymal epithelial cells cultures can be significantly decreased by rinsing the cultures twice a day with tissue culture medium during the first 3 days of culture. Epididymal epithelial cells isolated from pubertal and old adult rats reach confluency 6–7 days after plating.

Determination of Epididymal Epithelial Cells Growth and Viability. Epididymal epithelial cells from pubertal and old adult rats were seeded on glass coverslips and cultured in 60-mm plastic culture dishes in supplemented Eagle's medium as described above. After 24 hr of culture, the medium was replaced with fresh medium containing [*methyl*-^{3}H]thymidine (0.1 μCi/ml; 60 Ci/mmol, Schwarz/Mann; 1 Ci = 3.7×10^{10} becquerels). After continuous labeling for 1–6 days, cells on coverslips were fixed in methanol/acetic acid, 3:1, (vol/vol), air dried, and mounted with Permount (Fisher), cell side upward, on microscope slides. Slides were dipped in Kodak NTB-3 nuclear track emulsion. Coated slides were exposed at 4°C for 48 hr, developed in Kodak D-19 for 30 sec, rinsed in water, and fixed for 5 min in Kodak fixer. Slides were stained through the emulsion with 1% toluidine blue in 1% sodium borate for 3 min at room temperature. The mitotic index was determined in autoradiographic preparations counting a minimum of 1000 [^{3}H]thymidine-labeled cells; the numbers of metaphase, anaphase and telophase stages of mitosis were recorded.

Cell concentration and viability were determined during the first 6 days of culture. Cell viability was determined by dye exclusion after incubation of the sample in 0.4% trypan blue for 5 min at 32°C.

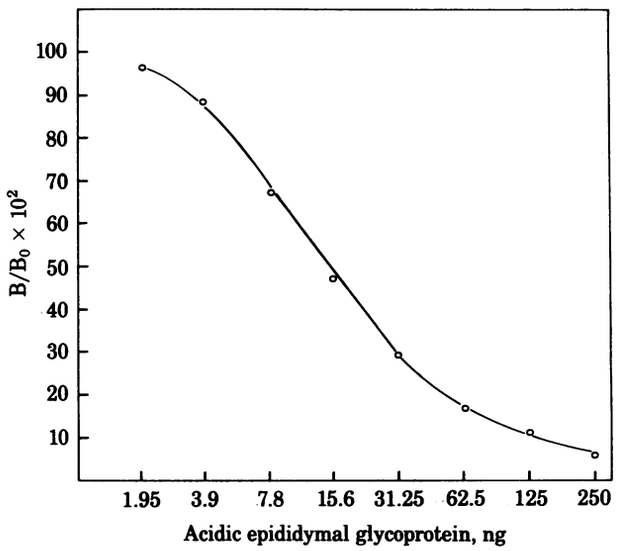

FIG. 2. Standard curve from acidic epididymal glycoprotein radioimmunoassay. Specific activity of tracer ^{125}I, 28.5 μCi/μg; final antiserum to dilution of acidic epididymal glycoprotein, 1:25,000. B, amount of tracer bound in the presence of unlabeled acidic epididymal glycoprotein; B_0, amount of tracer bound in absence of unlabeled acidic epididymal glycoprotein. A similar inhibition curve was obtained with epididymal cytosol.

Acidic Epididymal Glycoprotein Immunocytochemistry and Radioimmunoassay. The presence of acidic epididymal glycoprotein in cultured epididymal epithelial cells and its secretion into the medium were studied by immunocytochemistry and radioimmunoassay, respectively. Acidic epididymal glycoprotein was purified from rat epididymides as described (13). Antibody to acidic epididymal glycoprotein was raised in rabbits and purified by affinity chromatography on acidic epididymal glycoprotein-Sepharose. Purified acidic epididymal glycoprotein was labeled with ^{125}I according to the chloramine-T method (21) and the radioimmunoassay of medium samples was as described by Robyn *et al.* (22) with the double-antibody technique. Immunocytochemical localization of acidic epididymal glycoprotein in epididymal epithelial cells cultured on glass coverslips was carried out by the unlabeled antibody/enzyme-bridge technique (23). The specificity of acidic epididymal glycoprotein immunocytochemical staining was determined by the following criteria (c.f. ref. 24). "Method specificity" was evaluated by staining epididymal epithelial cells with increasing dilutions of anti-acidic epididymal glycoprotein antiserum. Optimal staining was obtained with dilutions (1:1000 to 1:10,000) of the primary antiserum. Because no staining was observed at higher dilutions (more than 1:100,000) it was possible to conclude that peroxidase staining depended on the acidic epididymal glycoprotein antiserum and not on subsequent reagents or on endogenous peroxidase activity. As an indication of antibody specificity, staining was eliminated when acidic epididymal glycoprotein antiserum was absorbed by preincubation with purified AEG or epididymis cytosol. The immunoperoxidase staining technique for cultured cells has been described (25). The standard curve of acidic epididymal glycoprotein radioimmunoassay is illustrated in Fig. 2.

RESULTS

Growth Pattern of Epididymal Epithelial Cells in Cultures. Epididymal epithelial cells isolated from either the whole epididymis or from caput, corpus, and cauda epididymis of 20- to

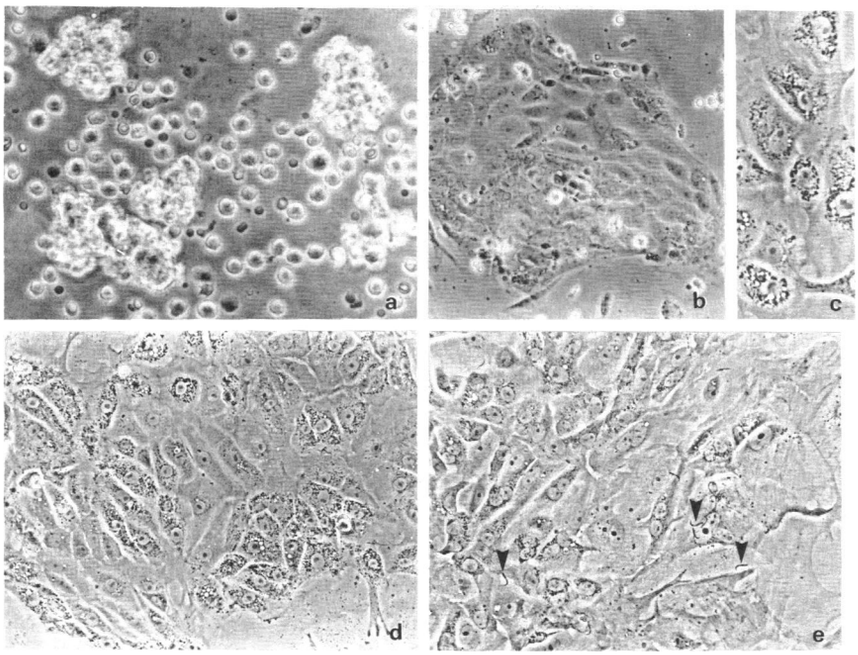

FIG. 3. Phase-contrast micrographs of epididymal epithelial cells cultured in a supplemented Eagle's medium. (*a–d*) Epididymal epithelial cells isolated from 20-day-old rats (whole epididymides); (*e*) epididymal epithelial cells isolated from 20-month-old rats (whole epididymides). (*a*) Epididymal epithelial cells at plating time. (×1060.) (*b* and *c*) At 29 hr after plating. (×560 and ×880, respectively.) (*d*) At 48 hr after plating. (×560.) (*e*) At 7 days after plating. Arrowheads indicate residual spermatozoa, mostly heads. Some of the epididymal epithelial cells display marked hypertrophy. (×660.)

22-day-old rats appeared as sphere-like aggregates and single cells at the time of plating (Fig. 3*a*). Within 29 hr in culture in supplemented Eagle's medium, epididymal epithelial cells attached to the substrate and gradually adopted a flattened configuration while arranging themselves into a colony-like growth pattern (Fig. 3*b*). At 48 hr after plating, nearly 80% of the cell colonies displayed a typical epithelial appearance defined by tightly packed, polygonal cells (Fig. 3*d*). DNA synthesis and mitotic activity were readily apparent at this time of culture (Fig. 4). In general, the mitotic index of cultured epididymal epithelial cells isolated from 20- to 22-day-old rats reached a maximum value (0.25) 3 days after plating. However, epididymal epithelial cells cultures established from caput epididymis had a slightly higher mitotic index (0.29) compared to cultures of cauda epididymis (0.22). Comparable mitotic indices were obtained 4 days after plating in epididymal epithelial cells cultures from 20-month-old rats (whole epididymis).

Most epididymal epithelial cells observed by phase-contrast microscopy displayed abundant cytoplasmic granules and vacuoles surrounding a spheric nucleus with a large nucleolus (Fig. 3 *c*, *d*, and *e*). A few binucleated cells could be observed in the preparations (Fig. 3*e*). The presence of cytoplasmic vacuoles in epididymal epithelial cells is consistent with the remarkable development of Golgi and endoplasmic reticulum cisternae observed in transmission electron micrographs (26) and with the synthesis and secretion of proteins as detected by gel electrophoresis of cell and medium samples (27).

As time progressed, epididymal epithelial cells located at the periphery of the aggregates became flattened and continued their migratory activity as they proliferated and underwent hypertrophy to form a typical monolayer configuration. Approximately 5–7 days after plating, epididymal epithelial cells reached confluency and maximal cell number (Fig. 5). Upon trypsinization of primary epididymal epithelial cells cultures and transfer to new culture flasks, epididymal epithelial cells rapidly attached to the substrate and resumed their proliferation rate in a growth pattern comparable to that of the primary cultures.

Immunocytochemical Localization of Acidic Epididymal Glycoprotein in Cultured Epididymal Epithelial Cells. It has been shown by Lea *et al.* (13) that acidic epididymal glycoprotein is secreted into the epididymal lumen by the principal cells of caput and corpus epididymides. Therefore, acidic epididymal glycoprotein was regarded as a suitable biological marker for the characterization of epididymal epithelial cells in cultures. Preparations of the caput epididymis of 20- to 22-day- and 20-month old rats were studied. Immunoperoxidase staining in the form of discrete clustered granules showed that acidic epididymal glycoprotein was localized in the cytoplasmic region adjacent to the nucleus (Fig. 6*a*). Acidic epididymal glycoprotein immunoreactivity was detected in about 80% of cultured epididymal epithelial cells isolated from caput epididymis. A similar pattern was observed in the principal cells lining the lumen of the ductus epididymis at about the middle portion of the caput (Fig. 6*b*). Acidic epididymal glycoprotein was localized in the apical region of the principal cells which are characterized by

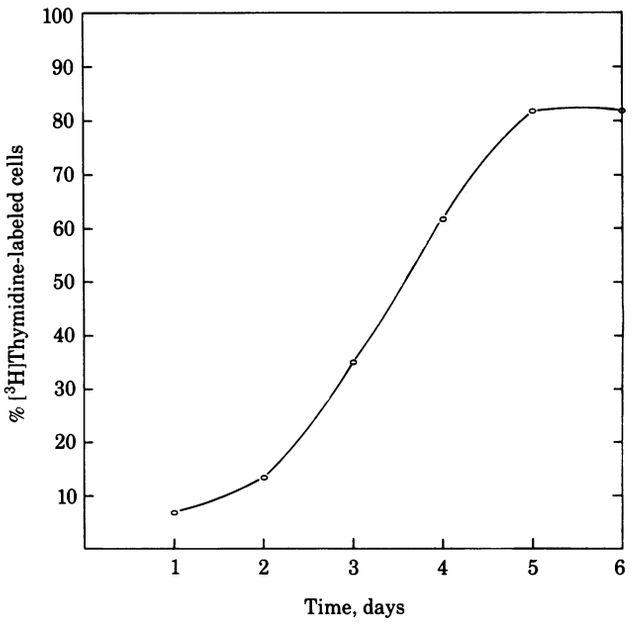

FIG. 4. Time course of [^{3}H]thymidine labeling of epididymal epithelial cells isolated from a 20-day-old rat. [^{3}H]Thymidine (0.1 μCi/ml) was added to the medium 1 day after plating. Maximal labeling percentage was observed 6 days after plating. Value per time point is the mean of triplicate experiments.

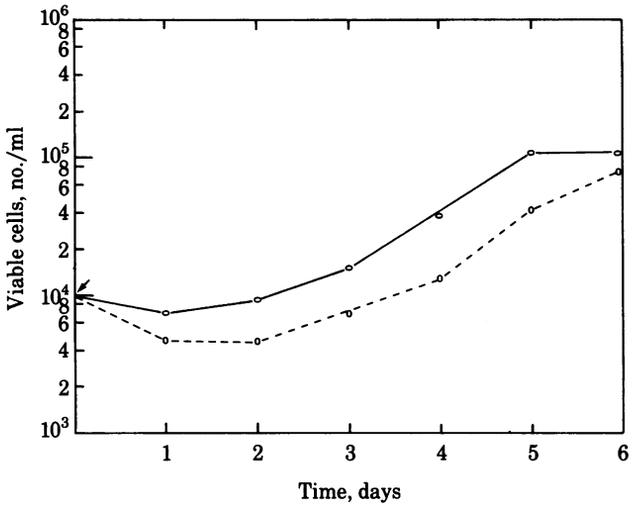

FIG. 5. Concentration of cultured epididymal epithelial cells from 20-day-old (○——○) and 20-month-old (○---○) rats. Cells (1×10^4/ml at time zero, arrow) were counted by the trypan blue exclusion test. Note the decline of viable cell concentration during the first day of culture and the continuous recovery afterward. The data represent the mean cell number in duplicate flasks.

the presence of a well-developed Golgi apparatus in electron microscopic preparations (28). Preliminary radioimmunoassays of acidic epididymal glycoprotein in the culture medium showed that it is secreted into the medium of cultured epididymal epithelial cells (20–100 ng/ml). These observations show that epididymal epithelial cells in culture maintain their differentiated protein synthetic and secretory activities.

DISCUSSION

A relatively simple cell isolation technique based on the consecutive use of two proteolytic enzymes, trypsin and collagenase, has been developed in our laboratory for the culture of rat epididymal epithelial cells in monolayers.

A considerable advantage of the epididymal epithelial cell culture method over organ culture of isolated segments of the epididymal duct is the direct evaluation of epididymal epithelial cell function without possible contributions of nonepithelial tissues. Thus far, *in vitro* experiments have been concerned with the isolation of epididymal epithelial cells by unit gravity sedimentation to study the physiology of these cells (15–18) and with the organ culture of isolated epididymal segments from sexually mature animals (29). Epididymal organ cultures are generally established after efferent duct ligation as a means of excluding spermatozoa and testicular fluid from the lumen of the tubules (29). Because it has been shown that the ligation procedure results in a rapid structural and functional regression of the epithelium of the proximal epididymal segments (30–32), the experimental data of epididymal epithelial cell function obtained after ligation should be interpreted with caution.

Several reports have stressed (*i*) the heterogeneity of the epididymal epithelial cell population with distinctive functional properties (33); (*ii*) the existence of several functional and structural segments or zones along the epididymal duct (19, 33, 34); and (*iii*) the change in chemical composition of the epididymal fluid from one region of the epididymis to another (35).

The heterogeneity of the epididymal epithelial cell population *in vivo* is of considerable interest because it poses intriguing questions about the identity of epididymal epithelial cells in cultures. Electron microscopic studies of the rat epididymis have distinguished at least five cell populations. Three cell types—principal cells, clear cells, and apical cells—occupy an adluminal epithelial location. The remaining two cell types—basal cells and halo cells—are found either adjacent to the basement membrane (basal cells) or interspersed among other cells at all levels of the epithelium (halo cells). The principal cell is the most common cell type. Ultrastructural (28, 33) and autoradiographic studies (36, 37) support the assumption that the principal cells are actively engaged in protein and glycoprotein secretory activities and, to a lesser extent, in the absorption of fluid and particulate material (38). There is general agreement that clear, apical, basal, and halo cells represent a lesser proportion of the total cell population of the epididymal epithelium of the sexually mature rat. These cells are involved in absorptive [clear cells (38)] and phagocytic activities [basal cells (1, 39)] as well as in the process of acidification and alkalinization [apical cells (40)] of the epididymal fluid. Halo cells correspond to intraepithelial, presumably migratory, leukocytes (28) or lymphocytes (41).

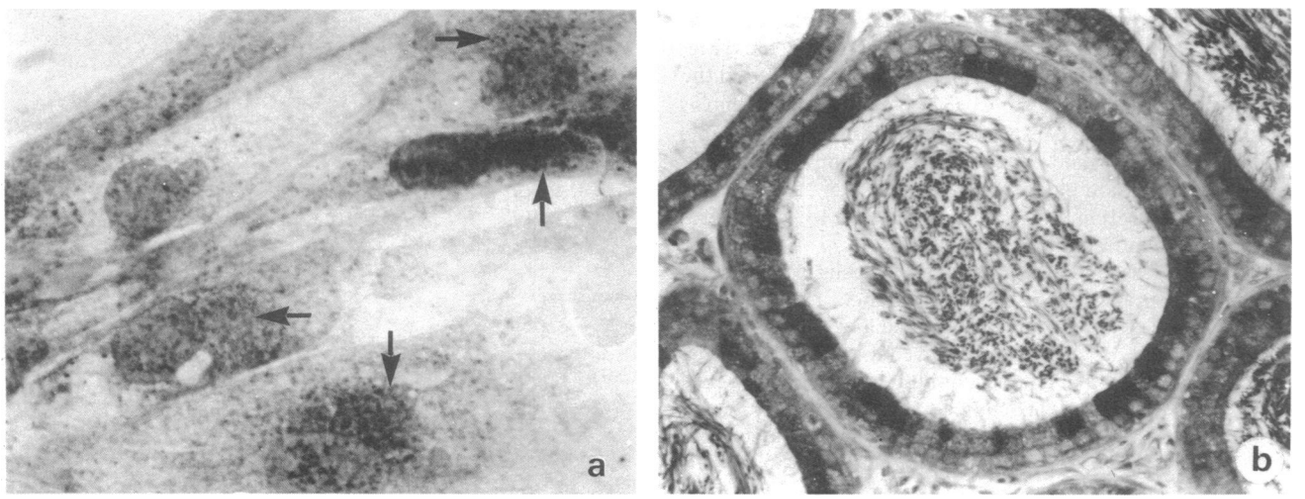

FIG. 6. Immunoperoxidase localization of acidic epididymal glycoprotein in epididymal epithelial cells isolated from a 20-day-old rat and in the caput epididymis distal to the initial segment. Purified acidic epididymal glycoprotein antiserum was diluted 1:1000. (*a*) Cultured (5 days) epididymal epithelial cells from caput epididymis. Clustered granules representing acidic epididymal glycoprotein immunoreactive content (arrows) are distributed in a juxtanuclear location. A few scattered peroxidase-stained granules can be observed throughout the cytoplasm. (×925.) (*b*) Caput epididymis. Acidic epididymal glycoprotein is present in a large number of principal cells; a few principal cells in the same epithelial layer lack acidic epididymal glycoprotein immunoreactivity. Spermatozoa in the lumen appear coated with acidic epididymal glycoprotein. (×700.)

Because the principal cells have been shown to synthesize and secrete acidic epididymal glycoprotein (13) and this cell type predominates in the rat epididymal epithelium, it was thought that a specific acidic epididymal glycoprotein antiserum could be utilized for immunocytochemical characterization of cells present in primary cultures established from whole epididymides or major anatomical segments of pubertal and old adult rats. The use of pubertal rats was based on the observation that the pubertal rat epididymal epithelium is active in the secretion of glycerylphosphorylcholine and sialic acid (42), two products associated with the functional maturation of the epididymis (43, 44) despite the absence of intraluminal spermatozoa. Old adult rats were used as an attempt to evaluate (*i*) the feasibility of culturing epididymal epithelial cells in the presence of spermatozoa and (*ii*) the proliferative activity *in vitro* of epididymal epithelial cells obtained from old rats known to have an insignificant turnover *in vivo* (45).

Quite clearly, a large proportion (80%) of epididymal epithelial cells in cultures established from pubertal and adult rats contain acidic epididymal glycoprotein which correlates with acidic epididymal glycoprotein immunoreactive sites in the principal cells of the epididymal epithelium *in vivo*. The finding that a small number of epididymal epithelial cells in culture are devoid of acidic epididymal glycoprotein immunoreactivity is in agreement with the variability in staining between individual principal cells *in vivo* (Fig. 6*b*; ref. 13) and suggests that the functional status of the cells is maintained *in vitro*. In fact, Killian *et al.* (18) reported variations in cell size and lipid content of principal cells in isolated rat epididymal cell fractions, findings which support the heterogeneous nature of the principal cell population *in vivo*.

Another relevant aspect of this study is the proliferative potential of epididymal epithelial cells in cultures. We have shown by autoradiographic analysis of [^{3}H]thymidine-labeled epididymal epithelial cells that DNA synthesis and mitotic cell division are regular features of cultured epididymal epithelial cells. [^{3}H]Thymidine-labeled cells exhibit structural characteristics similar to those observed in acidic epididymal glycoprotein immunoreactive epididymal epithelial cells. This correlative evidence leads to the conclusion that cultured epididymal epithelial cells established from both pubertal and adult rats can proliferate while retaining their capability to produce and secrete acidic epididymal glycoprotein. This proliferative behavior of cultured epididymal epithelial cells is in agreement with Clermont and Flannery's (45) autoradiographic results, but it is in contrast with their observation of a decline in the replicative activity *in vivo* with increasing age. Epididymal epithelial cells from mature rats apparently maintain the capacity to proliferate. When placed into primary culture they are released from some type of *in situ* inhibition or revert to an earlier stage of differentiation (34).

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1. Suzuki, F. & Glover, T. D. (1973) *J. Reprod. Fertil.* **35**, 584–585.
2. Hoffman, L. H., Jahad, N. & Orgebin-Crist, M.-C. (1976) *Cell Tissue Res.* **167**, 493–514.
3. Moore, H. D. M. & Bedford, J. M. (1979) *Anat. Rec.* **193**, 313–328..
4. Orgebin-Crist, M.-C. (1973) *J. Exp. Zool.* **185**, 301–310.
5. Lubicz-Nawrocki, C. M. & Glover, T. D. (1973) *J. Reprod. Fertil.* **34**, 315–329.
6. French, F. S. & Ritzen, E. M. (1973) *Endocrinology* **95**, 88–93.
7. Tindall, D. J., Hansson, V., Sar, M., Stumpf, W. E., French, F. S. & Nayfeh, S. N. (1974) *Endocrinology* **95**, 1119–1128.
8. Danzo, B. J., Cooper, T. G. & Orgebin-Crist, M.-C. (1977) *Biol. Reprod.* **17**, 64–77.
9. Purvis, K. & Hansson, V. (1978) *J. Reprod. Fertil.* **52**, 59–63.
10. Hoskins, D. D., Brandt, H. & Acott, T. S. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 2534–2542.
11. Bedford, J. M. & Cooper, G. W. (1978) in *Membrane Fusion: Cell Surface Review*, eds. Post, G. & Nicolson, G. L. (North-Holland, Amsterdam), Vol. 5, pp. 65–125.
12. Acott, T. S. & Hoskins, D. D. (1978) *J. Biol. Chem.* **253**, 6744–6750.
13. Lea, O. A., Petrusz, P. & French, F. S. (1978) *Int. J. Androl. Suppl.* **2**, 592–605.
14. Olson, G. E. & Hamilton, D. W. (1978) *Biol. Reprod.* **19**, 26–35.
15. Killian, G. J. & Amann, R. P. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 225.
16. Brooks, D. E. (1975) *Andrologia* **7**, 241–253.
17. Killian, G. J., Amman, R. P. & Snyder, J. (1979) *Biol. Reprod.* **15**, 266–279.
18. Killian, G. J., Snyder, J. & Amman, R. P. (1977) *Cell Tissue Res.* **183**, 371–378.
19. Reid, B. L. & Cleland, K. (1957) *Austr. J. Zool.* **7**, 22–38.
20. Webber, M. M. (1979) *In Vitro* **15**, 967–982.
21. Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123.
22. Robyn, C., L'Hermite, M., Petrusz, P. & Diczfalusy, E. (1971) *Acta Endocrinol.* **67**, 417–433.
23. Petrusz, P., DiMeo, P., Ordronneau, P., Weaver, R. C. & Keefer, D. A. (1975) *Histochemistry* **46**, 9–26.
24. Petrusz, P., Sar, M., Ordronneau, P. & DiMeo, P. (1976) *J. Histochem. Cytochem.* **24**, 1110–1112.
25. Kierszenbaum, A. L., Feldman, M., Lea, O., Spruill, W. A., Tres, L. L., Petrusz, P. & French, F. S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5322–5326.
26. White, M. G., Tres. L. L. & Kierszenbaum, A. L. (1979) *Anat. Rec.* **193**, 719–720.
27. Kierszenbaum, A. L. & Huang, Y.-S. (1978) *J. Cell Biol.* **79**, 179a.
28. Hoffer, A. P., Hamilton, D. W. & Fawcett, D. W. (1973) *Anat. Rec.* **175**, 169–202.
29. Blaquier, J. A. (1973) *Biochem. Biophys. Res. Commun.* **52**, 1177–1183.
30. Gustafsson, B. (1966) *Acta Vet. Scand. Suppl.* **17**, 1.
31. Prasad, M. R. N., Rajalakshmi, M., Gupta, G. & Karkun, T. (1973) *J. Reprod. Fertil. Suppl.* **18**, 215–222.
32. Fawcett, D. W. & Hoffer, A. P. (1979) *Biol. Reprod.* **20**, 162–181.
33. Hamilton, D. W. (1975) in *Handbook of Physiology Endocrinology*, eds. Astwood, E. B. & Greep. R. O. (Am. Physiol. Soc., Bethesda, MD), Vol. 5, pp. 259–301.
34. Sun, E. L. & Flickinger, C. J. (1979) *Am. J. Anat.* **154**, 27–56.
35. Levine, N. & Marsh, D. J. (1971) *J. Physiol. (London)* **213**, 557–570.
36. Neutra, M. & Leblond, C. P. (1966) *J. Cell Biol.* **30**, 137–150.
37. Flickinger, C. J. & Stuart, M. W. (1978) *J. Cell Biol.* **79**, 370a.
38. Moore, H. D. M. & Bedford, J. M. (1979) *Anat. Rec.* **193**, 293–312.
39. Grant, J. H. (1958) *Proc. Soc. Study Fertil.* **10**, 95–101.
40. Cohen, J. P., Hoffer, A. P. & Rosen, S. (1976) *Biol. Reprod.* **14**, 339–346.
41. Dym, M. & Romrell, L. J. (1975) *J. Reprod. Fertil.* **42**, 1–7.
42. Setty, B. S. & Jehan, Q. (1977) *J. Reprod. Fertil.* **49**, 317–322.
43. Dawson, R. M. & Rowlands, I. W. (1959) *Q. J. Exp. Physiol.* **44**, 26–34.
44. Rajalakshmi, M. & Prasad, M. R. N. (1969) *J. Endocrinol.* **44**, 379–385.
45. Clermont, Y. & Flannery, J. (1970) *Biol. Reprod.* **3**, 283–292.